ABSTRACT

Title of Dissertation / Thesis: **SUBSTRATE SPECIFICITY AND MOLECULAR CHARACTERIZATION OF PAH-DEGRADING ANAEROBIC ENRICHMENT CULTURES UNDER METHANOGENIC CONDITIONS.**

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Over the past several years, this lab has cultivated enriched anaerobic mixed cultures capable of degrading the low-molecular-weight polycyclic aromatic hydrocarbons (PAHs), naphthalene or phenanthrene, as a sole carbon source. Characterization of the enrichment cultures by 16S rDNA analysis revealed two unique populations, though they were initiated from the same sediment source. This study set out to determine the ability of these highly enriched communities to utilize a new carbon source, and to identify the effect of this shift on the population profile. The phenanthrene-degrading culture demonstrated significant naphthalene-degrading activity; however, the naphthalene-degrading culture was unable to degrade phenanthrene. Molecular characterization of these cultures showed a single species common to both original enrichment cultures and dominating in the population able to degrade both substrates. These results suggest that very few members of the mixed cultures participate in the degradation of PAHs, whereas the remaining members live off the metabolic byproducts.

SUBSTRATE SPECIFICITY AND MOLECULAR CHARACTERIZATION OF PAH-DEGRADING ANAEROBIC ENRICHMENT CULTURES UNDER METHANOGENIC CONDITIONS.

By

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Chapter 1: Background and Introduction

PAHs in the Environment

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing two or more aromatic rings, classified by the number of rings present. Compounds with two to three rings are considered low molecular weight PAHs, whereas those with four or more are high molecular weight. The U. S. Environmental Protection Agency (EPA) classified PAHs as priority pollutants. Many exhibit carcinogenic, mutagenic and teratogenic properties at acute doses, and a wide range of toxicity with chronic exposure.[1]

Low background levels of PAHs occur ubiquitously in the environment resulting from biosynthesis.[1,2] Certain marine bacteria, fungi and phytoplankton synthesize aromatics or quinines, which are easily reduced to PAHs in the environment.[1] Thiele and Brummer[2] demonstrated PAH bioformation from plant materials in waterlogged anoxic soils. With this constant concentration of PAH in the environment, several microbial populations have evolved to resist their toxicity and even to degrade these compounds. However, PAHs are also major components of crude and refined fossil fuels, and are introduced to the environment in large quantities via oil spills, or run-off from industrial activities[1].

Once in the marine environment, they tend to adsorb to suspended particulate matter and phytoplankton and are quickly transported to the sea floor. Though they may return to the water column through a small amount of leaching, their low solubility primarily results in PAH accumulation in the anoxic sediments of the benthic region.[1,3] Though biodegradation has been shown as an effective remediation

strategy for PAHs in other environments, available electron acceptors are quickly depleted in subsurface sediments, leaving methanogenic conditions at the contaminated site.

Methanogens and Methanogenesis

The study of methanogenesis dates back to 1776, with Allesandro Volta's discovery of "combustible air" emanating from marshy sediments. In 1868, Bechamp associated the formation of this phenomenon with the generation of methane from larger carbon compounds by microbial activities. The 1900s marked an explosion of methanogenic research, which still continues through today. The interest in methanogens stems from their role in the global methane cycle, and their potential contribution to global warming. The continued study has revealed a wide diversity of physical, environmental and biochemical characteristics, including physiology and metabolic systems, giving rise to several considerable taxonomic changes.[4]

Methanogens are members of the domain *Archaea*, along with hyperthermophiles, halophiles and other extremophilic microbes. Many are the strictest of anaerobes, where even incidental exposure to oxygen can be lethal, and thrive under extreme redox conditions. They generate methane through the metabolism of simple single-carbon molecules, such as carbon dioxide, acetate, or methylated compounds. With this narrow substrate range, methanogens rely on the metabolic byproducts of larger molecules released by fermentative bacteria. Thus, they often exist in concert with sulfate reducing bacteria, which can produce acetate

from a variety of organic compounds. This relationship provides a greater energy yield than the complete oxidation by sulfate reducers themselves[4].

Previous PAH-Degradation Studies

Previous research in this lab revealed the first reported case of degradation of two low molecular weight PAHs, naphthalene and phenanthrene, under methanogenic conditions[5]. These results were obtained using a method of selective enrichment coupled with molecular modeling (SEMM) approach described by Holoman et al.[6]

Sediments from a PAH-contaminated area of Baltimore Harbor were collected anaerobically and transferred to minimal media cultures. Pyrene, phenanthrene and naphthalene were added individually to the cultures as a sole carbon source and the disappearance of the PAH was monitored using gas chromatography with a flame ionization detector (GC-FID). The phenanthrene and naphthalene cultures showed PAH degradation coupled with methane production, without a lag period.[5]

Community structure of each PAH-degrading enrichment culture was established with 16S rDNA analysis using a procedure described by Holoman et al.[6] Briefly, cells were withdrawn from live cultures and lysed to extract genomic DNA. PCR amplification was performed with universal and *Archaeal* primers. DNA fragments were cloned, sequenced, and analyzed to establish community profiles.

The results of Chang's molecular modeling analysis showed unique community profiles for the two enrichment cultures, each containing a mixture of ten or more anaerobic bacterial and methanogenic species.[5] The naphthalene-degrading cultures contained several bacterial species similar to the *Firmicutes* (Gram-positive bacteria) and the methanogenic family *Methanoccus*. The phenanthrene-degrading

cultures contained homologs of the *Bacillus/Clostridium* subgroup and archaeal species similar to *Methanosarcinaceae sp*. Over the past three years, these cultures have been periodically refed and transferred to fresh media to enrich for methanogenic PAH-degrading communities and minimize the sediment content of the cultures.

Previous Substrate Specificity Studies

Previous substrate specificity studies have focused primarily on pure cultures of aerobic bacteria that degrade BTEX compounds (benzene, toluene, ethylbenzene and xylenes) and low molecular weight PAHs.[7,8,9,10,11,12,13] In general, the studies found that aromatic-degrading microorganisms can also utilize smaller aromatic compounds. Gulesoy and Alvarez[9] found that recalcitrant compounds were more easily degraded when present in mixtures. Previous work with anaerobic (sulfate reducing) PAH degradation indicates that degradation rates increase as molecular weight increases and rates were enhanced when compounds were fed in mixtures.[12] With this agreement between results of sulfate-reducing cultures and aerobes, it is reasonable to expect that the results of methanogenic substrate specificity experiments will be similar to those performed under aerobic conditions.

Objectives of this Research

This is the first recorded study on the substrate specificity of PAH-degrading microorganisms under methanogenic conditions. The objectives of this study were threefold; (1) to confirm the previous results of methanogenic PAH degradation, (2) to explore the substrate specificity of the enriched PAH-degrading cultures, and (3) to

characterize the community structure of these cultures after a shift in their sole carbon source.

Objectives (1) and (2) were achieved by repeating the experiments performed by Chang, with the addition of the shift in carbon source (see Table 1). Objective (3) was achieved using the molecular monitoring approach previously described. Once the population profiles were determined by 16S rRNA analysis, they were compared to determine the affect of an alternative carbon source on the community structure. Specifically, enrichment cultures able to degrade alternative compounds were examined to determine if the degradative activity is likely the result of a population shift or adaptation of the original enriched population.

Chapter 2: Methods and Materials

Enrichment Cultures

The cultures used in this study were inoculated by the enriched phenanthrene and naphthalene degraders cultivated by Chang. They are the fourth generation of sequential transfers from the original sediment sample and are virtually sediment-free. All cultures were grown in Estuarine media containing 8.4 g/L NaCl, 3.95 g/L $MgCl_2 \cdot 6H_20$, 0.27 g/L KCl, 0.05 g/L $CaCl_2 \cdot 2H_20$, 0.5 g/L NH_4Cl, 3.0 g/L Na_2CO_3, 1.12 g/L Na_2HPO_4, 0.25 g/L cysteine, 1.0 ml/L resazurin, 10 ml/L vitamin solution, and 10 ml/L trace solution. Concentrated HCl was titrated to pH 6.8 and media was dispensed under an anaerobic (4:1 N_2-CO_2) atmosphere. All chemicals were obtained from Sigma Chemical.

In an anaerobic glove box, 5 ml aliquots of the appropriate inoculum were transferred to 100 ml serum vials containing 50 ml of fresh media. Naphthalene and phenanthrene were solubilized in acetone. Each culture was individually fed either naphthalene or phenanthrene stock solution, according to Table 1, giving a final PAH concentration of 200 μM. Each culture was labeled with a two-letter designation, the first letter indicating the enrichment culture's native substrate and the second letter denoting the carbon-source used in this study (see table 1).

Culture Label	Native Carbon Source	Experimental Carbon Source
P-P	Phenanthrene	Phenanthrene
N-N	Naphthalene	Naphthalene
N-P	Naphthalene	Phenanthrene
P-N	Phenanthrene	Naphthalene

Table 1. Description of cultures used in this study. Native Carbon Source refers to the PAH used to generate enrichment cultures. Experimental Carbon Source refers to PAH used in this study.

The vials were capped with butyl rubber stoppers and crimped with aluminum seals. Sodium sulfate (1% vol/vol) was added to each vial by syringe as a reducing agent. Controls were prepared in the same manner and autoclaved three times over the following week. All samples, including the autoclaved controls, were prepared in triplicate. Cultures were incubated at 30° C in the dark without shaking.

GC Analysis of PAH Degradation

Biweekly, 0.7 ml of culture was withdrawn form each vial and added to an equal volume of hexane. When biphenyl was used as an internal standard, it was added at a final concentration of 100 μM. The vials were capped and shaken overnight then centrifuged for one minute. 2 μL of supernatant was injected into a Hewlett-Packard gas chromatograph (HP 5890A) connected to a Hewlett- Packard flame ionization detector (FID) (HP 7673A). The CG column was a 30m DB5 capillary column(J&W Scientific) with an inner diameter or 0.25 mm. The injector and detector temperatures were 280°C and 300°C, respectively. The column was maintained at 50°C for 1 minute, increased at a rate of 30°C per minute, then held at a final temperature of 300°C for 3 minutes. The resulting chromatograph peaks were analyzed by a Hewlett-Packard integrator. PAH concentrations were calculated from the resulting peak area. The first two data points were calculated with a linear calibration plot. All subsequent data points were calculated using biphenyl as an internal standard. Data points for triplicate cultures were averaged and plotted in Microsoft® Excel 2002.

Molecular Monitoring

Extraction and Amplification of Genomic DNA

Once PAH-degradation activity was confirmed, cultures were prepared for genomic DNA extraction. 1 mL of each culture was withdrawn by syringe and centrifuged for 3 minutes at 13000 rpm. 750 μL of the supernatant was discarded and the process was repeated for each of the triplicate cultures, giving a final sample volume of 750 μL with a 4x concentration of cells. The samples were resuspended then frozen for one hour to lyse the cells. They were then thawed, vortexed, and allowed to settle to remove suspended sediment particles.

The concentrated samples were immediately used as a template for genomic PCR amplification. Amplification was performed using universal primers, 519F (5'-CGA CA/CG CCG CGG TAA TA/TC-3') and 1406R (5'-ACG GGC GGT GTG TA/GC-3'). These primers enclose a 900bp segment of prokaryotic 16S rDNA. The PCR cycle temperature program consisted of an initial denaturation step of 1½ minutes at 94°, followed by 30 cycles of 30 seconds at 94°, 30 seconds at 55 and 20 seconds at 72°, plus a final extension step of 5 minutes at 72°. PCR effectiveness was verified by electrophoresis at 75V for 40 minutes on a 1% agarose gel.

Transformation, and Reamplification of DNA Fragments

Following amplification, 120μl of fresh PCR product was purified using the QIAQuick PCR Purification Kit (QIAGEN 28104), according to the manufacturer's instructions. DNA fragments were eluted in 50 μL of water then cloned using the TOPO TA Cloning Kit (Invitrogen K4500-01). The purified PCR product was inserted into the pCR2.1 TOPO vector, supplied with the kit. This vector contains

kanamycin and ampicillin resistance genes, as well as a *lacZ* reporter gene as a locus for the PCR product insertion. The plasmid was then transformed into chemically competent *E. coli* TOP10 cells, also provided with the kit. Transformed cells were plated on LB agar containing 40µl/g kanamycin, and X-Gal and incubated overnight at 30°C in the dark. The following day, 96 white colonies were randomly selected and restreaked on LB/kanamycin selective plates using sterile toothpicks, then incubated overnight at 30°C in the dark. Individual colonies were transferred to 3 ml of LB media and incubated overnight at 37°C with shaking at 150 rpm. Overnight cultures were reamplified with the same primers, using the colony PCR temperature program of 3 minutes at 95°, followed by 40 cycles of 30 seconds at 95°, 30 seconds at 55° and 30 seconds at 72°, then a final 5 minutes at 72°.

RFLP Analysis

Colony PCR products were individually digested overnight with restriction enzymes HaeIII and HhaI. Digests were separated by electrophoresis on a 3% Trevi-Gel (TreviGen 9804-050-P) at 35V for 3 hours. The gel was then stained with ethidium bromide for one hour, destained in water, and viewed under UV light. Images of RFLP bands were captured by a Stratagene Eagle Eye II fluoroimager, running EagleSight (v3.2) software.

The images were inspected to identify common RFLP patterns. A pattern was considered to be “well-resolved” if bands produced by either digest could be visually discerned within the range of the molecular weight marker, which provides a comparison standard between 147 and 900bp on the 3% gel. Unique patterns were

identified and designated by a two-digit label, i.e. x.y, where x represents a unique pattern from the HaeIII digests, and y represents a HhaI digestion pattern.

Since the host *E. coli* strain also contains a region of 16S rDNA that can be amplified by the primers, there is a risk of amplifying *E. coli* genomic 16S rDNA, despite the high copy number of the TOPO plasmid. To eliminate these patterns the *E. coli* 16S rDNA sequence was retrieved from NCBI, and the region enclosed by the primers was submitted to New England Biolab's Restriction Enzyme Database (REBASE) website for theoretical digestion by HaeIII and HhaI. Colonies exhibiting this RFLP pattern were neglected.

Determining Sterility of Autoclaved Controls

To determine the sterility of the autoclaved controls, a rich 10x nutrient mixture was created consisting of 5.5 mg/mL each of starch, tryptone, peptone, sodium acetate, glucose, and yeast extract, 22.2 μL/mL methanol and ethanol, and .55M TMA. 4.5 mL of cultures P-P, P-P*, N-N, N-N*, P-N and P-N*, as well as 4.5 mL of fresh media were transferred to serum vial in an anaerobic glove box. 0.5 mL of nutrient mixture was added to each culture. Immediately, 0.5 mL was extracted by syringe and transferred to a cuvette for OD600 measurement to determine a baseline for each culture. The cultures were incubated at 30° in the dark. Optical density measurements were repeated after 8 days to assess growth.

Chapter 3: Results

PAH Degradation

Phenanthrene Degradation

Figure 1 shows the average concentrations of phenanthrene in each culture over time. The concentration of phenanthrene in the P-P active cultures (see table 1 for explanation of culture labels) decreased by an average of 155 μM over a period of 123 days (fig. 1A), whereas the average phenanthrene concentration in the autoclaved controls (indicated by the dashed lines) decreased by only 28 μM over the course of the study.

The naphthalene-degrading cultures (N-P) showed an average decrease in phenanthrene concentration of 26.5 μM in the active cultures and 37.3 μM in the autoclaved controls (fig. 1B). This data reveals virtually no decrease in the active or control cultures as shown by the pink line in figure 1.

The P-N cultures initially contained a low concentration of phenanthrene carried over from the inoculation. The average phenanthrene concentration in these cultures decreased by 30.4 μM (fig. 1C).

Naphthalene Degradation

Average concentrations of naphthalene are shown in figure 2. The N-N active cultures (fig. 2A) showed a decrease in average naphthalene concentration of 247.5 μM over 116 days. In the N-N controls, naphthalene concentrations decreased by an average of 171.5 μM. Over the course of the study, the average concentration in P-N naphthlane concentration decreased by 156.8 μM (fig. 2B). The N-P cultures (fig. 2C)

also contained a small amount of naphthalene from the innoculum. This concentration decreased by 17.75 μM.

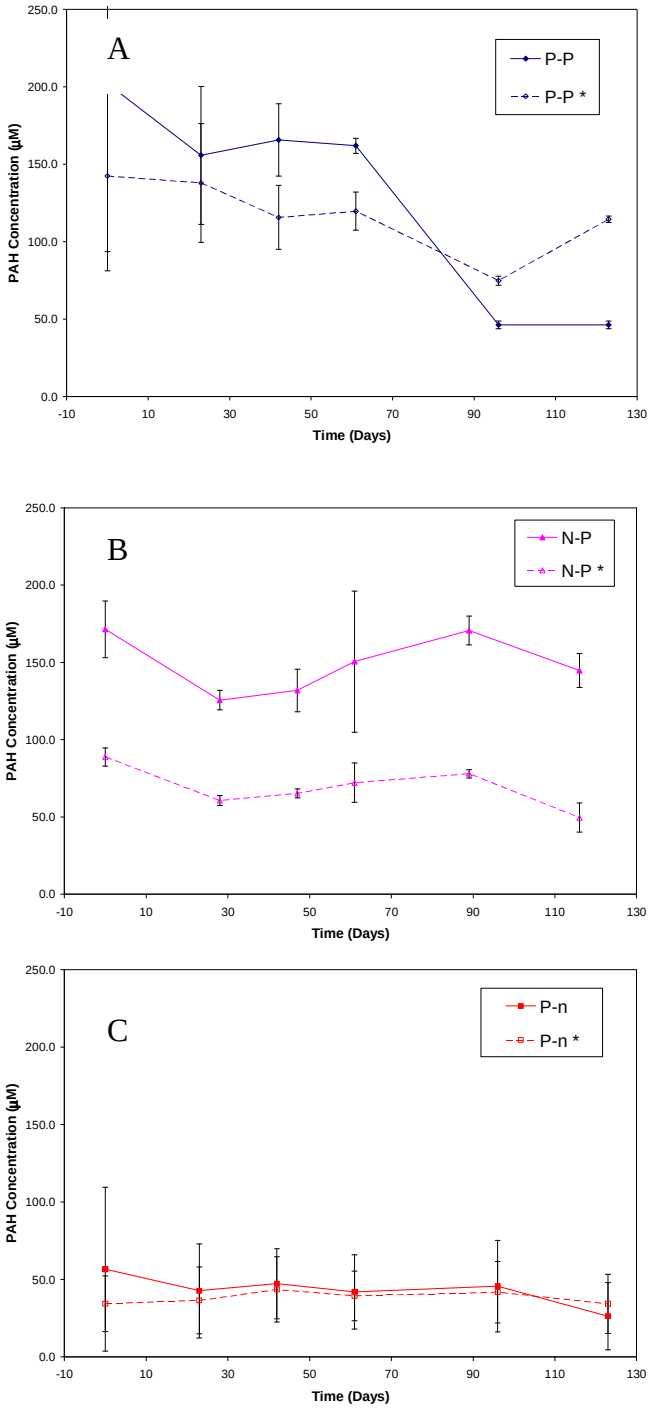


Figure 1: Phenanthrene degradation by (A) P-P, (B) N-P, and (C) P-N over time, measured in µM. Symbols indicate the average of triplicate cultures. Error bars represent one standard deviation. Autoclaved controls are shown by open symbols and dashed lines.

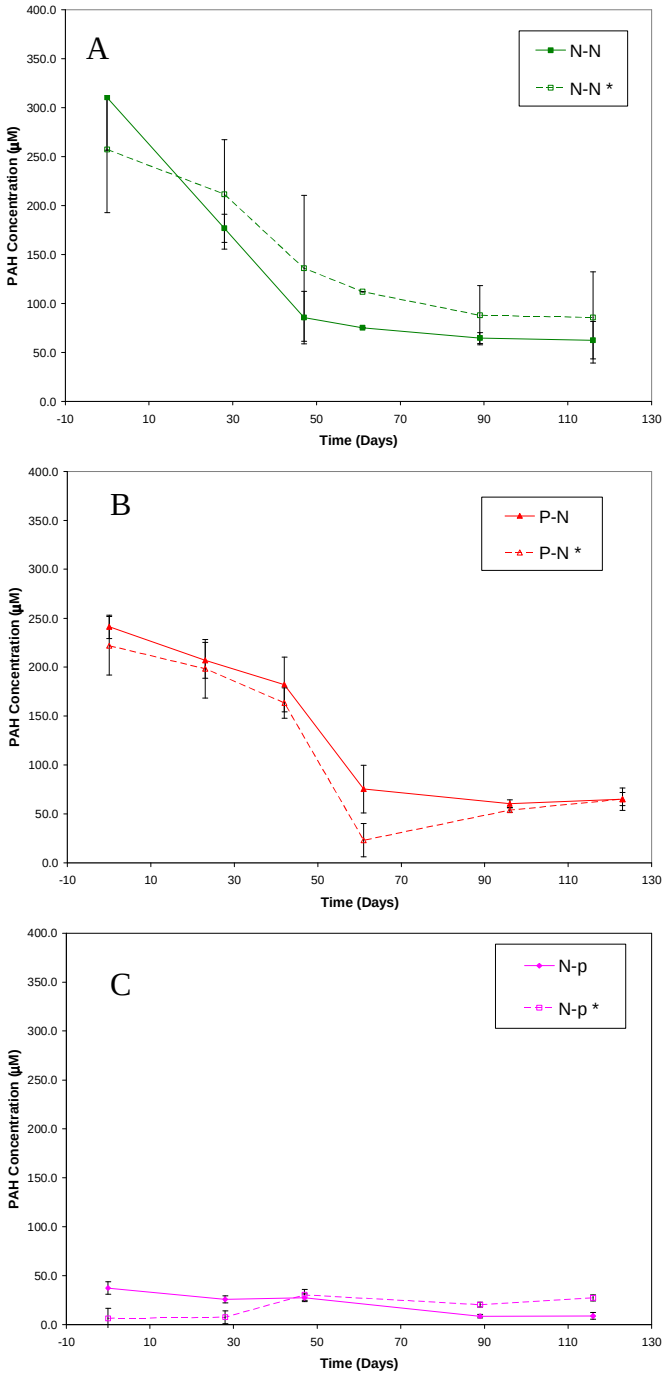


Figure 2: Naphthalene degradation by (A) N-N, (B) P-N and (C) N-P. Symbols indicate the average of triplicate cultures. Error bars represent one standard deviation. Autoclaved controls are shown by open symbols and dashed lines.

To assess the sterility of the controls, 5 mL samples of the P-P, P-N, and N-N active and control cultures were fed a rich nutrient mixture to stimulate growth. Growth was approximated by optical density measurements at 600 nm. Results of these measurements are shown in figure 3. After 8 days, P-P and N-N active cultures showed a significant increase in OD_{600}, whereas P-N shows only a minimal increase. Of the control cultures, the P-P* showed an OD gradient similar to the fresh media. Both P-N* and N-N* showed a moderate increase in OD_{600}.

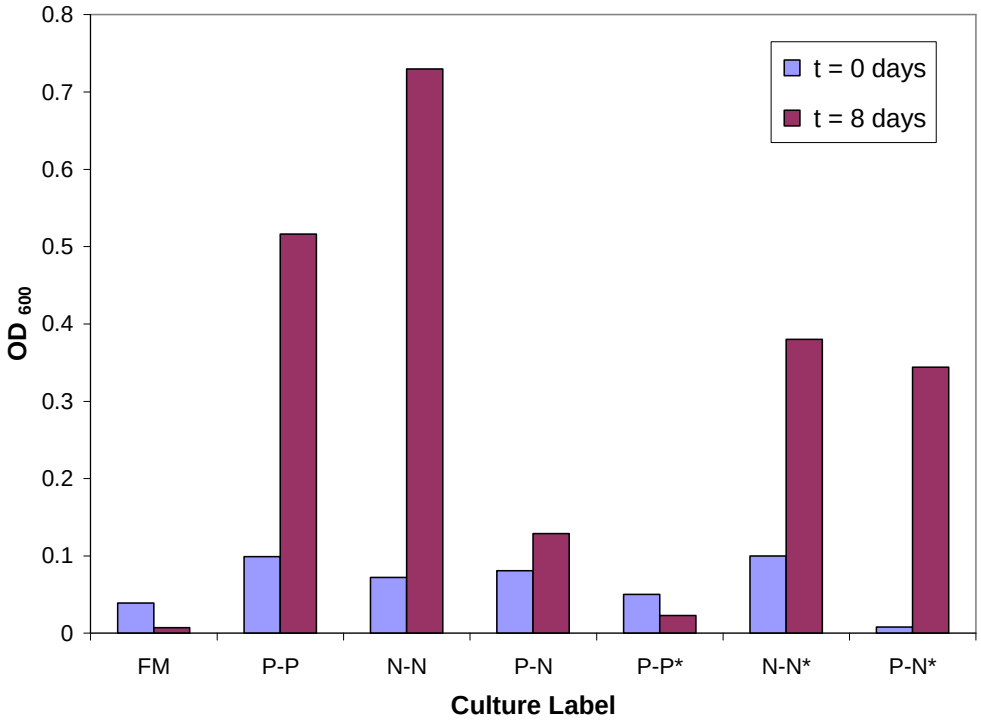


Figure 3: Optical density of P-P, N-N and P-N cultures and fresh media at 600 nm. Autoclaved controls are indicated by asterisks.

Molecular Monitoring

Native Carbon Source Populations (P-P and N-N)

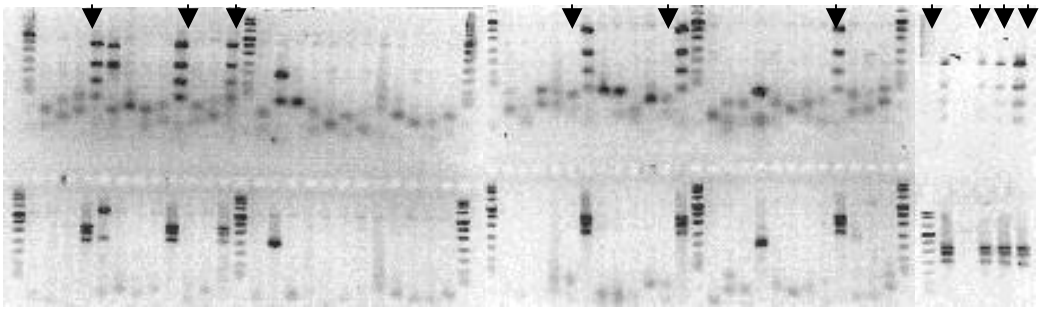
To investigate the effect of a phenanthrene-degrading enrichment culture utilizing naphthalene as a sole carbon source, the community profiles were determined by 16S rDNA analysis. The P-P and N-N cultures were initially examined for comparison. Amplification of the 96 P-P colonies gave 53 positive PCR products. When the fragments were digested by HaeIII and HhaI, 19 resulted in RFLP bands with sufficient resolution for analysis. Six unique patterns were identified, with two being represented by more than one colony. The dominant pattern, designated 1.1, occurred in nine of the 19 lanes. The second most abundant pattern, 4.4, was repeated 3 times.

Amplification of the N-N colonies gave 37 positive PCR products. Of these, 23 resulted in well-resolved RFLP patterns after enzyme digestion, with six being unique and four of these occurring more than once. Pattern 1.1 dominated in this culture as well, with seven colonies, along with pattern 5.5, also being represented by seven colonies. Pattern 3.1 appeared five times and pattern 0.4 twice. With the exception of pattern 1.1, no other patterns were seen in both cultures.

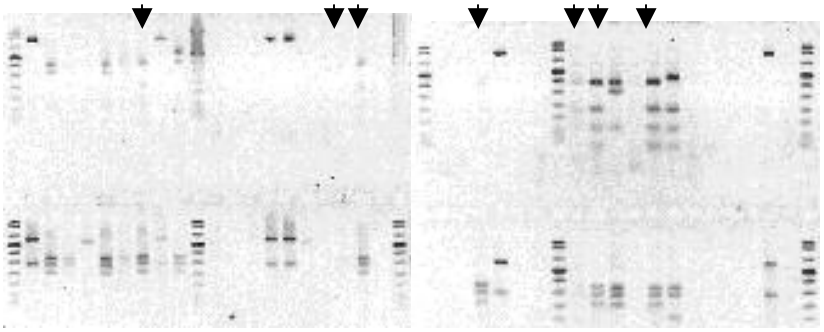
Alternative Carbon Source Population (P-N)

Amplification of the P-N cultures gave 53 positive clones and 41 well-resolved RFLPs. Of these, eight were unique and three appeared more than once. RFLP pattern 1.1 occurred 30 times in this culture. This was the only pattern that was represented in the native cultures. Patterns 1.2 and 1.3, which share the same HaeIII pattern with 1.1, appeared six and two times, respectively, and did not appear in the P-P or N-N cultures.

A



B



C

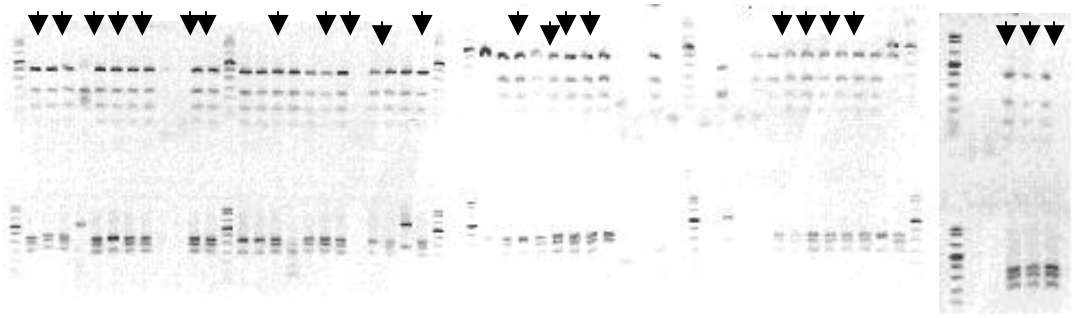


Figure 4: RFLP Patterns of (A) P-P, (B) N-N, and (C) P-N. In each gel, HaeIII digests are loaded on the top row and HhaI digests on the bottom. Each pattern is designated by a two-digit label in the format "x.y", where x indicates a unique HaeIII pattern, and y indicates a unique HhaI pattern. Arrows mark lanes containing RFLP patterns designated "1.1"

Chapter 4: Discussion

Anaerobic Degradation of PAHs

The phenanthrene-degrading enrichment cultures showed the greatest diversity in PAH degradation. This is not surprising, as previous studies have shown that aerobic pure cultures able to degrade aromatics can generally degrade other aromatic compounds of lower molecular weight. This trend is confirmed by the inability of the naphthalene-degraders to degrade phenanthrene.

It is interesting to observe the response of the P-N and N-P cultures to the residual PAH concentrations carried over from the initial inoculum cultures. These data are at the limit of reliable interpolation either by linear calibration or by use of an internal standard, thus they are not conclusive, but both cultures appear to demonstrate only slight degradation of their native substrates with respect to the controls. Since either culture should be expected to efficiently degrade its own native carbon source, this result raises some interesting questions. The decreased degradative activity could indicate a possible threshold concentration required to stimulate metabolism. However, concurrent research in this lab is investigating PAH degradation at low concentrations, similar to those seen in the residual cultures, with successful results.[14] Alternatively, the limited degradation of native substrates could be the result of substrate interactions, wherein the more readily available naphthalene in the P-N cultures could inhibit uptake of the phenanthrene. Similarly, the presence of phenanthrene in the N-P cultures could limit phenanthrene degradation

Of greatest concern is the apparent ability of the autoclaved controls to degrade naphthalene as well. This trend has been observed in other experiments in

this lab[14]. Possible explanations include chemical interactions between naphthalene and the estuarine media, sorption of naphthalene to the vial, stopper or sediments, evaporation of naphthalene during sampling, or biological activity in the control cultures.

When supplied with a rich nutrient mixture, both the P-N and N-N controls showed significant microbial growth. While the possibility of contamination during sampling for GC analysis would seem most likely, each of the triplicate cultures shows the same trend for all three systems. The probability of randomly contaminating all three of the P-N and N-N controls and none of the P-P controls is relatively low. The biological activity in the autoclaved controls may also suggest that at least one of the naphthalene degraders is resistant to sterilization. Although controls were autoclaved on three different days, spore-forming microbes may have survived, returning to an active state during incubation. This possibility can be further investigated by pasteurizing the control cultures and reassess for growth and PAH degradation. Whether this phenomenon is a result of physical, chemical or biological interactions with naphthalene, or laboratory contamination this issue bears further investigation.

Community Profiles of PAH-Degrading Enrichment Cultures

The cultures showed considerably less community diversity than those in Chang's study, likely a response to the selective pressure of the enrichment scheme. The dominance of pattern 1.1 in the P-N cultures suggests that this organism plays a key role in PAH degradation, outcompeting other organisms for the substrate. This

hypothesis is further supported by the appearance of this pattern in both the N-N and P-P cultures.

Based on Chang's original work, identifying an RFLP pattern common to all three cultures was unexpected. Chang's results showed no RFLP type in both phenanthrene and naphthalene degrading cultures. Based on these results, it is important to recognize that the abundance of an RFLP pattern in a culture does not necessarily represent the abundance of an organism in the population. This method carries a considerable potential for bias which can arise at several points throughout the process. In sampling of the cultures, organisms which are adhered to the walls of the vial or sorbed to the sediments would be underrepresented. Though swirling the vials prior to withdrawing samples may dislodge microbes adhered to the vial, those closely associated with the sediments could be missed entirely, as sediment particles were strictly avoided in preparing PCR reactions. Within the PCR amplification process, there exists bias based on the selection of the primers. Though this region of microbial DNA is highly conserved, the primer sequences are not universal, and may exclude significant members of the population. The cloning process may also introduce bias, as some fragments may be more easily inserted into the vector than others. Additionally, there may be some selective pressure on transformed *E. coli* cells containing plasmids with certain inserted DNA fragments.

Additionally, a unique RFLP pattern does not necessarily represent a unique organism. Several species may randomly exhibit the same pattern when digested by a particular restriction enzyme. This is seen in the P-N cultures where three RFLP types (1.1, 1.2 and 1.3) share the same HaeIII digest patterns, but differ in the HhaI

patterns. The potential for this erroneous assumption is reduced by digestion with the second enzyme. Unique organisms could be further resolved by a third digestion, either with another enzyme or by the combination of the two already in use.

Many of these factors exhibit a consistent bias across different samples, thus their cumulative effect would make this method useful mainly for relative or qualitative analysis rather than absolute conclusions about the community diversity of the samples.

Chapter 5: Conclusions and Recommendations

The results presented here contribute to the larger study of methanogenic biodegradation of PAHs in the environment initiated by Chang. Over time, the PAH-degrading enrichment cultures cultivated from that study have been selected for growth on a very specific substrate range. Natural microbial populations, however, constantly respond to periodic shifts in their environment. Organisms that are unable to adapt to the introduction of new carbon sources, especially toxic compounds will be eliminated. While background levels of aromatics in the benthic zone are relatively constant, industrial activities cause intermittent spikes in PAH levels. With the recalcitrance of these compounds, a contaminated site will become highly enriched for organisms capable of degrading these compounds. As the primary substrate is depleted, individuals with the ability to utilize other compounds will outcompete those with less diverse metabolic abilities. This study confirms the trend seen in aerobic pure cultures that a shift to a lighter substrate is favored over a shift to higher molecular weight compound. This multiplicity is accomplished by the emerging dominance of the more diverse metabolic organisms.

The ultimate goal of this research is to identify physical, chemical and environmental factors that can be exploited to induce or enhance contaminant biodegradation by indigenous organisms. Continued work in this area presents the potential for further research opportunities in a wide variety of areas.

The mechanism for the degradation of naphthalene and phenanthrene is still unknown. Biodegradation pathways commonly proceed through intermediates of the TCA cycle. According to the Kyoto Encyclopedia of Genes and Genomes metabolic

pathway database (www.kegg.com), the degradation pathway of both naphthalene and phenanthrene will proceed through either pyruvate or catechol. Identification of metabolic intermediates produced by these cultures would suggest which pathway dominates, or suggest a novel mechanism.

Along with the identification of intermediates and elucidation of the organic reaction mechanism, another critical element of a full biodegradation pathway would include the identification, isolation and characterization of metabolic enzymes. Investigation of aerobic PAH degradation revealed that many of the enzymes involved are components of the *tod* operon, responsible for toluene degradation.[12] Previous study with biodegradation of aromatics indicates that compounds are more readily degraded when present in mixtures, especially when toluene is present. The enhanced degradation of many aromatics in the presence of toluene suggests that induction of this pathway is involved in metabolism of aromatic compounds. As PAHs arising from both natural and human sources commonly occur in mixtures, the effect of substrate interactions in the induction or repression of degradation is a crucial element to the broad application of a bioremediation strategy. Though Chang's study did not show pyrene degradation under methanogenic conditions, examination of PAH mixtures could reveal conditions under which this degradation is possible.

Though it is unlikely that the inconclusive data regarding the recalcitrance of the residual PAHs from native cultures was a result of a minimum substrate concentration required to initiate degradation, an effective concentration range likely exists. Organisms that are highly enriched for growth on a single carbon source may

succumb to competitive pressure when that substrate is depleted. Identification of a minimum contaminant concentration necessary for sustaining growth can provide a limit of effectiveness for biodegradation. The increased toxicity of aromatics in acute doses also suggests a maximum concentration. The cultures studied here grew in contaminant doses up to 250μM. Though this concentration is orders of magnitude higher than the ambient levels, localized spikes often occur as a result of anthropogenic activity. To be effective in bioremediation strategies, the ability of these microbes to withstand such spikes must be investigated.

The cumulative contribution of these factors, along with the continued current research in this area, will bring the pollution response industry closer to identifying feasible bioremediation strategies for these highly toxic organic compounds.

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